REMARKS / ARGUMENTS

As requested by the Examiner in the communication mailed July 25, 2006, enclosed pleased find a substitute declaration.

In response to the Examiner's rejection of the claims under Section 112, all prior claims have been cancelled. In their stead, applicant now presents claims, 7 through 13. It is respectfully submitted that such newly presented claim complys fully with Section 112. In particular, applicant has adopted the Examiner's recommendation regarding the structure of the method of treatment claims.

1. Introduction

Potent activities have been published for celastrol and pristimerin in various enzymatic and cellular systems. Notable is the demonstration that these compounds can independently induce the Heat Shock Response (HSR) *in vitro* at low concentrations. Both compounds, however, have shown serious cellular toxicity (q.v.) which has stifled their development as useful drugs.

Celastrol and pristimerin are related as acid and ester respectively on the same pentacyclic nortriterpene framework. A comparative analysis of celastrol, pristimerin and their derivatives in relation to their ability to induce HSR has led to the discovery that potent HSR induction can be retained despite significant chemical change in these molecules. The abilities of compounds possessing the chemical functionality found in celastrol, but devoid of its topography have been assessed. In that assessment, it has been discovered that a quinone or a quinone methide moiety does not alone confer the ability to induce HSR.

This invention relates to the discovery of compounds, structurally related to celastrol and pristimerin, that retain the ability to induce HSR and thereby serve as useful therapeutic candidates, in addition to being useful in chemical libraries for high-throughput screening.

2. Therapeutic Significance of the Heat Shock Response

The HSR is an endogenous defensive mechanism that is activated by stress situations. It is an ordered genetic response to various physiological stimuli that results in the biosynthesis and release of a barrage of proteins (the Hsp proteins) that have a wide range of physiological functions. The response is conserved in evolution from prokaryotes to humans. Stimuli for the response include, but are not limited to, elevated temperature, inflammation, ischemia, and stress. HSR is self regulated at the transcriptional level through heat shock transcription factors (HSF) [L Pirkkala, P Nykanen, and L Sistonen, FASEB J 15:1118 (2001)]. HSF-1 is the factor that is best characterized, and shown to be essential for HSR. HSF-1 stimulates the production of Hsp70 and launches the Hsp cascade. HSR is managed by a feedback mechanism involving the attenuation of the HSR cascade by Hsp90. Hsp90 binds to HSF-1 and inactivates it.

The Hsp proteins are organized by molecular weight, such as, Hsp90, Hsp70, Hsp60, etc. Many Hsp proteins serve as molecular chaperones which participate in the correct assembly and folding of other proteins critical to cell function, and the degradation of those that are misfolded or otherwise malformed. These functions are very important in ensuring the correct three dimensional structure of signal proteins, receptor components and enzymes that are critical to cell survival. Misfolding can, for example, result in the deposition of protein complexes which are centrally linked to many inflammatory and neurodegenerative disease processes. Current experimental and clinical observations underscore the potential of HSR modulators as therapeutic tools for diseases of protein conformation [SD Westerheide and RI Morimoto, J Biol Chem 280:33097 (2005)].

The HSR response involves more than the chaperone role. For example, NK-kappaB is a ubiquitous inducible transcription factor that is involved in inflammation by promoting transcription of numerous inflammatory factors and cytokines. HSF-1 has been shown to serve as a protective agent against the severity of human acute pancreatitis through inactivation of NK-kappaB, the activation of cytoprotective genes and the downregulation of inflammatory genes [RI Morimoto and MG Santoro, Nat Biotechnol 16:833 (1998); MG Santoro Biochem Pharmacol 59:55 (2000); DA O'Reilly, JR Roberts, MT Cartmell, AG Demaine and AN Kingsnorth, J Pancreas 7:174 (2006)]. The ability of HSR to block angiotensin-II induced hypertension and inflammation of the aorta, is similarly attributed to

inactivation of NK-kappaB [Y Chen, BM Ross and RW Currie, Cell Stress and Chaperones 9:99 (2004)].

HSR has been shown to enhance myocardial ischemic tolerance [R Cornelussen, W Spiering, JH Webers, LG DeBriun, RS Reneman, GT van der Vusse and LH Subeckx, Am J Physiol Heart Circ Physiol, 267: H1941 (1994)] and post-ischemia recovery [RW Currie, M Karmazyn, M Kloc, and K Maller, Circ Res 63:543 (1988)]. Infart size is also limited in isolated perfused rabbit heart [DM Walker, E Pasini, S Kucukoglu, MS Marber, E Iloidromitis, R Ferrari and DM Yellon, Cardiovasc Res 27:1962 (1993)]. Mediation of cardioprotection by HSR has been associated with activation of protein kinase C [D Tekin, L Xi, T Zhao, MI Tejero-Taldo, S Atlura and RC Kukreja, Am J Physiol Heart Circ Physiol 281:H523 (2001)] and the opening of ATP-sensitive K channels [TJ Pell, DM Yellon, RW Goodwin and G Baxter, Cardiovasc Drugs Ther 11:679 (1997)]. HSR also protects cells from apoptotic and necrotic death. The generation of OH radicals from mitochondrial superoxide in cardiac cells is attenuated by HSR through the enhancement of Mn superoxide dismutase and subsequent inhibition of aconitase [G Ilangouan, CD Venkatakrishnam, A Bratasz, S Osinbowale, AJ Candounel, JL Zweier and P Kuppusamy, Am J Physiol Cell Physiol 290:C313 (2006)].

Tumor Necrosis Factor (TNF) is a potent antineoplastic agent. However, its use has been restricted to susceptible tumors in isolated tissue and limbs due to its toxicity to the organism as a whole [DL Franker and HR Alexander, Import Adv Oncol 1994:179]. It has been shown that mice previously exposed to heat shock (42°C for 20 min) blocked the systemic effect of TNF while retaining the antineoplastic effect on the tumor. [W VanMolle et al.,Immunity 16:685 (2002)]. This important finding draws attention to the potential of HSR as an endogenous synergist in antineoplastic therapy [M Leist and M Jaattela, Nature Med 8:667 (2002)].

Another example of synergy is found in the ability of HSR to enhance the antineoplastic effect of replication-competent adenovirus [YS Haviv, JL Blackwell, H Li, M Wang, X Lei and DT Curiel, Cancer Res 61:8361 (2001)].

Other examples of the therapeutic scope of HSR have been provided by studies addressing the effects of overexpresion of HSR through small molecule stimulation.

3. Small Molecule Induction of HSR

The induction of HSR in humans can have beneficial effects in a wide variety of disorders. Unfortunately, duplicating HSR in humans is a difficult task. Even a sauna at 100°C is ineffective in eliciting HSR [M Leist and M Jaattela, Nature Med 8:667 (2002)]. The discovery that certain small molecules could initiate HSR, however, launched a search for therapeutically acceptable compounds that would have the capacity to induce HSR on demand and serve as a novel "broad spectrum" approach to the alleviation of human diseases.

Hsp90, as noted above, is the heat shock protein responsible for shutting down HSR by binding and inactivation HSF-1. Radicicol and geldanamycin are Hsp90 inhibitors with antineoplastic and antifungal activities [L Whitesell, R Bagatell and R Falsey], Geldanamycin has been the inhibitor most studied. Its profile and development as a therapeutic agent is relevant to the significance of this invention.

Geldanamycin is a macrolide possessing benzoquinone and carbamate moieties—both potently bioactive groups. It promotes HSR by binding to Hsp90 and reducing its co-immunoprecipitation with HSF-1 [Human Mol Genetics 10:1307 (2001);13:1389 (2004)]. HSF-1 is thereby released and the HSR cascade initiated. Geldanamycin has been demonstrated to be anti-inflammatory *in vivo* in the carrageenan paw edema assay in rats by interference in the binding of Hsp90 at steroid receptor sites. This result identified Hsp90 as critical for pathways involved in CPE inflammation and suggests a therapeutic rationale for the enhancement of HSR by specific inhibitors of Hsp90 and their potential as anti-inflammatory drugs [M Bucci, F Roviezzo, C Cicala, WC Sessa and G Cirino, Br J Pharmacol 131:13 (2000)].

It has also been shown that treatment of mammalian cells with geldanamycin at nanomolar concentrations supresses huntingtin protein aggregation by inducing HSF-1. The authors conclude that these findings may provide the basis for the development of a novel pharmacotherapy for Huntington's disease and related disorders. [A Sittler, R Lurz, G Lueder, J Priller, H Lehrach, MK Hayer-Hartl, FU Hartl, EE Wanker, Hum Mol Genet 10:1307 (2001)]

The utility of geldanamycin has been further demonstrated experimentally in rats by its ability to block focal ischemia in the brain [A Lu, R Ran, S Parmentier-Batteur, A Nee and FR Sharp, J Neurochem 81:355 (2002)]

Geldanamycin was also considered as a candidate for antineoplastic therapy by virtue of its ability to block Hsp90 induced activation of client proteins associated with apoptosis and oncogenesis.[Y Miyata Curr Pharm Des 11:1131 (2005)]. Unfortunately, geldanamycin proved to be a potent liver toxin [JG Supko, RL Hickman, MR Grever and L Malspeis, Cancer Chemother Pharmacol 36:305 (1995)] and development was halted. Modification of the structure of geldanamycin has led to the discovery and development of a derivative of geldanamycin (17-allylaminogeldanamycin; NSC-330507D; 17-AAG) that has one tenth the toxicity of its parent. 17-AAG is in clinical development.

4. Stimulation of HSR by Celastrol and its Derivatives

Celastrol and several of its derivatives rapidly induce HSR without physical stress. They activate HSF-1 which triggers the HSR, greatly increasing the cellular content of Hsp70 and lower homologs. Notable is the activity of the dihydro-diacetate derivative, which approaches the same potency of celastrol itself. [SD Westerheide, JD Bosman, BN Mbadugha, TL Kawahara, G Matsumoto, S Kim, W Gu, JP Devlin, RB Silverman, RL Morimoto, J Biol Chem 279:56053 (2004)]. The authors also report that a suboptimal dose of celastrol together with low temperature heat exposure—both of which do not produce HSR—produce maximal HSR. Celastrol does not have any direct effect on protein folding or Hsp70 activities.

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is selectively toxic to the cells in the *substantia nigra*, and is capable of producing virtually all the signs and symptoms of idiopathic Parkinson's disease in both experimental animal and humans (as a drug of abuse). MPTP has been used as a model of Parkinson's disease. Celastrol significantly attenuated the loss of dopaminergic neurons and the depletion of dopamine induced by MPTP. [C Cleren, NY Calingasan, J Chen and MF Beal, J Neurochem 94:995 (2005)]. In the same paper it was shown that celastrol reduces the striated lesions induced in rats by 3-nitropropionic acid—a response that serves as a model of Huntington's disease.

5. Toxicity of Celastrol

The toxicity of quinones has been well established. It is associated with arylation of cellular nucleophiles (e.g., cisteinyl thiols) through Michael Addition, and the catalytic reduction of oxygen to superoxide and other reactive oxygen species through redox cycling. [JM Karczewski, JG Peters and J Noordhoek, Biochem Pharmacol 57:27 (1999); CE Rodriguez, M Shinyashiki, J Froines, CY Rong, JM Fukoto and AK Cho, Toxicology 201:185 (2004); Arylation induces endoplasmic reticulum stress by activating the pancreatic ER kinase (PERK) signaling pathway [X Wan g, B Thomas, R Sachdeva, L Arteburn, L Frye, PG Hatcher, DG Cornwell and J Ma, PNAS 103:3604 (2006)]. Quinone methides are chemically equivalent to quinones and it is reasonable to assume that toxicity will be manifest in an analogous manner. The mechanisms involved with the toxicity of celastrol, therefore, can be associated at least in part with arylation and the generation of reactive oxygen species.

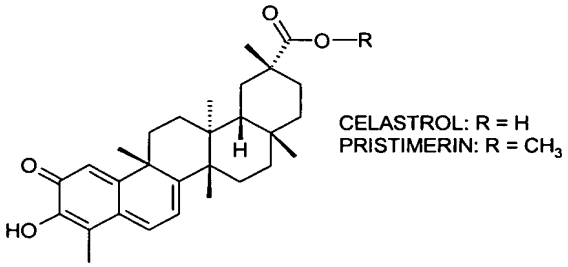
Another toxic manifestation of celastrol has recently been discovered. Celastrol has been shown [H Sun, X Liu, Q Xiong, S Shikano and M Li, J Biol Chem 281:5877 (2006)] to be a potent inhibitor of potassium channel conductance. Its mechanism is manifest as a reduction of channel density on the cell surface and blockage of ion conductance. Those effects are found at concentrations of celastrol equivalent to or lower than that required to enhance HSF-1. This finding essentially puts a hold on the development of celastrol as an agent useful in the therapy of human disease. It, nevertheless, provides a focus for comparison with analogs of celastrol that retain the ability to enhance HSR while reducing this manifestation of toxicity.

6. The Invention

This invention relates to the design and development of derivatives of celastrol that will retain the benefits of HSR induction and yet are substantially reduced in toxicity. The toxicity of celastrol negates its therapeutic potential in the same manner that the development of geldanamycin (*vide supra*) was halted. As a medicinal chemist and one skilled in the art, I conclude that such development is realistic in scope and supported by the specification of the subject application.

The invention describes a significant change in the character of celastrol by reduction of the quinine methide to the corresponding catechol moiety, and the derivatization of that product to a form that is devoid of quinine character. Such character can be restored through normal cellular processes. These derivatives have been prepared and shown to retain the ability to induce HSR.

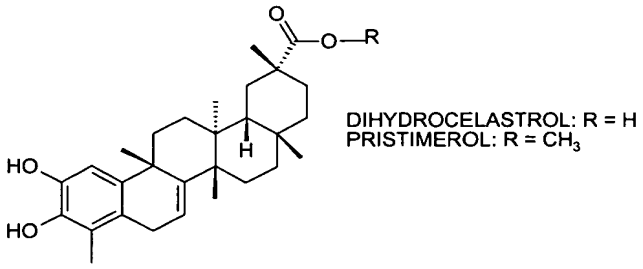
Celastrol and pristimerin are both classified as pentacyclic nortriterpenes (**I**). Both compounds also possess as hydroxyquinone methide moiety extended by one additional double bond.



I

Pentacyclic triterpenes represent a large family of natural products that are generally devoid of significant biological activity. They are highly lipophylic and sparsely substituted with polar groups—the most common being a hydroxyl group (usually at C-3), and/or a carboxylic acid moiety. The introduction of reactive groups such as epoxides, unsaturated ketones, quinine, quinine methides, and/or the appendage of sugar moieties, results in the creation of molecular species that have increased polarity and the ability to combine wih receptors, enzymes and other biologically significant sites.

The extended hydroxyquinone methide moiety in both celastrol and pristimerin is a feature that confers potent biological properties to these nortriterpene scaffolds. That moiety can be reduced to the dihydro derivatives (**II**) with sodium borohydride according to published procedures [K Nakanishi, Y Takahashi and H Budzikiewicz, J Org Chem 30:1729 (1965)]. These derivatives can be readily converted back to the parent by aerial oxidation in solution at room temperature.

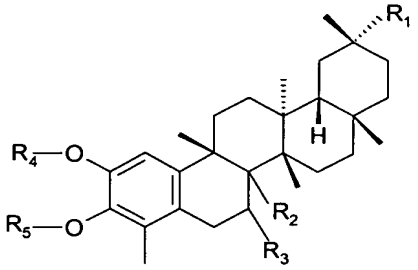


II

Acetylation of dihydrocelastrol with acetic anhydride/pyridine, and recrystallization of the product from toluene/hexane yields dihydrocelastrol diacetate as pale yellow crystals, mp 224-226°C (lit mp 211-212°C). 1H NMR ($CDCl_3$) 8 Me's, 1 double bond proton (5.7 ppm), 1 aromatic proton (6.9 ppm), 17 aliphatic protons, one exchangeable proton; ^{13}C NMR ($CDCl_3$) 3o=c-o, 8 aromatic and double bond signals, 22 aliphatic signals; MS: M-H @ 535, M+Cl @ 581, and M+Na @ 559. Purity (>95%) was confirmed by HPLC (UV & light scattering detectors).

Dihydropristimerin (pristimerol) is similary prepared and converted to the diacetate derivative.

The invention encompasses compounds of the general formulae **III**.



III

- wherein R_1 is H, CH_2OH, COOH, CH_2OCOR wherein R is C-1 to C-12 alkyl, carboxyalkyl, carboxyalkenyl, alkoxycarbonylalkyl, or aminoalkyl;
- wherein R_2 and R_3 are individually H or OH, or together a double bond or epoxide; and

- wherein R_4 and R_5 are individually H, lower acyl, or lower alkyl, or together are a substituted or unsubstituted methylene or ethylene, -CO-, -COCO-, or $-SO_2-$.

In view of this, it is respectfully submitted that the compounds herein described and the subject of the newly added claims present both scaffolds for the development of biologically active compounds that will retain activity and also provide less toxicity than is possible with either celastrol or pristimerin, in addition to being candidates for development in and of themselves. As is described in the specification at page three (3), such compounds have demonstrated biological activity. Additionally, as outlined above, the toxicity profiles are much more favorable than either celastrol or pristimerin.

In view of the foregoing, it is respectfully submitted that the subject application is in condition for allowance and such favorable action at an early date is earnestly solicited.

Respectfully submitted,

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on November 25, 2006

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